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13. <u>Abstract</u> . In an effort explore the feasibility of a baculovirus recombinant dengue-1 (DEN 1) virus vaccine, a portion of the envelope (E) glycoprotein gene from DEN 1 virus was cloned and expressed in baculovirus (<i>Autographa californica</i> nuclear polyhedrosis virus, AcNPV). The recombinant construct contains 107 nucleotides from the 3' terminus of the M gene which encodes a hydrophobic signal peptide and extends through the first 1245 nucleotides of E, terminating 243 nucleotides before the 3' terminus of E. When the recombinant was grown in <i>Spodoptera frugiperda</i> cells, about 1 mg of E antigen was made per 10 ⁹ /cells. The recombinant E antigen reacted with E protein-specific monoclonal antibodies and stimulated the production of DEN 1 virus neutralizing antibody in BALB/c mice. Mice immunized with the recombinant E antigen or with heat-inactivated DEN 1 virus were protected significantly against lethal DEN 1 virus challenge. A dose response effect was observed with increasing amounts of recombinant antigen leading to increased survival.			
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DENGUE-1 VIRUS ENVELOPE GLYCOPROTEIN GENE EXPRESSED IN RECOMBINANT BACULOVIRUS ELICITS VIRUS-NEUTRALIZING ANTIBODY IN MICE AND PROTECTS THEM FROM VIRUS CHALLENGE

ROBERT PUTNAK, ROBERT FEIGHNY, JEANNE BURROUS, MARK COCHRAN,
CRAIG HACKETT, GALE SMITH, AND CHARLES HOKE

*Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, DC;
MicroGeneSys Inc., West Haven, Connecticut*

Abstract. In order to test the feasibility of baculovirus (*Autographa californica* nuclear polyhedrosis virus, AcNPV) expression vectors for making immunogens against dengue-1 (DEN-1) virus, a portion of the envelope (E) glycoprotein gene of DEN-1 virus was cloned and expressed. The recombinant baculovirus contains 107 nucleotides from the 3' terminus of the DEN-1 matrix (M) gene, which encodes a hydrophobic signal peptide and extends through the first 1,245 nucleotides of E, terminating 243 nucleotides before the 3' terminus of E. When the recombinant virus was grown in *Spodoptera frugiperda* cells, about 1 mg of E antigen was made per 10⁹ cells. Recombinant E antigen reacted with E protein-specific monoclonal antibodies and stimulated production of DEN-1 virus neutralizing antibody in BALB/c mice. Mice immunized with recombinant E antigen or with heat-inactivated DEN-1 virus were protected significantly against lethal DEN-1 virus challenge. A dose/response effect was observed, with increasing amounts of recombinant antigen leading to increased survival. These results demonstrate the utility of baculovirus for producing immunogens against DEN-1 virus.

Dengue-1 (DEN-1) virus is one of four serotypes (DEN-1, DEN-2, DEN-3, and DEN-4) of the dengue virus group in the family *flaviviridae*.¹ The virus, which is transmitted by *Aedes aegypti* mosquitoes, generally causes an acute, febrile illness.² However, more serious illnesses, dengue hemorrhagic fever, and shock syndrome, can also occur.³⁻⁴ Thus far, no effective vaccines against dengue viruses have been licensed for human use, although several live attenuated vaccines have been used experimentally in man with varying success.⁵⁻⁷

Dengue is an enveloped, positive-stranded, RNA virus about 50 nanometers in diameter.^{8,9} Like the prototype flavivirus yellow fever, all of its viral proteins are encoded by a single, long, open-reading frame of about 10.5 kilobases arranged as follows: 5' C-prM/M-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 3'.¹⁰⁻¹⁵ Virion structural proteins (capsid, pre-matrix/matrix and envelope) are encoded at the 5' end, followed by nonstructural (NS) proteins encoded by the remainder of the genome. Proteins are produced by co-translational proteolytic processing by host cell signal peptidase¹⁶⁻²⁰ and putative viral proteinases.^{21,22}

The envelope (E) glycoprotein is the major sur-

face protein of the flavivirus; it functions in cell attachment and contains neutralization epitopes thought to be important in antiviral immunity.²³⁻²⁷ The E protein is preceded in the polyprotein by a hydrophobic amino acid segment at the carboxyl-terminus of prM/M that functions as a cleavable transmembrane sequence.¹¹ It terminates with two hydrophobic segments at the carboxyl-terminus, which may serve as a membrane anchor.¹¹⁻²⁰

Knowledge of flavivirus gene structure, coupled with positive results obtained with authentic E and NS1 proteins as subunit immunogens in animal models^{28,29} has led to the use of recombinant cDNA technology for producing these proteins. Baculovirus vectors have attracted much interest because of the potentially high levels of foreign gene expression that can be attained,³⁰ and for their lack of pathogenicity for humans. Foreign genes are expressed from the baculovirus polyhedrin promoter,^{31,32} and recombinant proteins appear to be correctly processed and glycosylated.³²⁻³⁴

Baculovirus has been used for expression of flaviviruses, including DEN-4, in which a polygenic region, C-prM-E-NS1-NS2A, yielded antigens that conferred complete protection to

mice,³⁵ and Japanese encephalitis (JE), in which polygenic regions, C-prM-E, prM-E-NS1-NS2A-NS2B, or E by itself, yielded antigens that elicited neutralizing antibody^{34, 36} and protected mice.³⁴ These results point to flavivirus E protein made by baculovirus as a potential recombinant immunogen.

We report herein that an E gene cDNA sequence derived from DEN-1, when expressed in baculovirus, synthesized protein that was antigenic, elicited DEN-1 virus-neutralizing antibody in mice, and provided significant protection against mortality from DEN-1 encephalitis.

MATERIALS AND METHODS

Construction of a baculovirus-recombinant E glycoprotein gene and its expression in Spodoptera frugiperda cells

RNA obtained from a Western Pacific strain of DEN-1 virus isolated on Nauru Island³⁷ in 1974 was cloned and sequenced as previously described.¹² A cDNA clone extending from nucleotides 58 to 2559¹² was used for the construction of a recombinant E gene fragment by digestion with *Bst*NI (cuts at nucleotides 801 and 2150). The restriction fragment was treated with bacteriophage T4 DNA polymerase and all four deoxynucleoside triphosphates to make blunt ends, and cloned into the unique *Sma*I site of a baculovirus (*Autographa californica* nuclear polyhedrosis virus, AcNPV) recombination vector, pAcMGS, described previously.³⁴ Recombinant baculovirus was obtained by co-transfecting *S. frugiperda* insect cells in monolayer culture with the recombination vector and wild-type baculovirus DNA. As a result of homologous recombination, the baculovirus polyhedrin gene was replaced by the DEN-1 cDNA insert, rendering recombinants polyhedrin negative.³⁴

Viruses forming polyhedrin occlusion body-negative (clear) plaques were selected and plaque-purified three times. Recombinant virus was used to infect *S. frugiperda* cells in suspension cultures. Infected cells were pelleted by low speed centrifugation and lysed by Dounce homogenization. Cell lysates were electrophoresed on polyacrylamide gels containing sodium dodecylsulfate (SDS),³⁸ and either stained with Coomassie blue R-250 or electrophoretically transferred to nitrocellulose. Antigenic bands were detected by a modification of Western blotting,³⁹ using spe-

cific antiserum (mouse hyperimmune ascitic fluid, MHAF, 1:200) against DEN-1, followed by goat anti-mouse immunoglobulin (instead of protein A used in the original method) labeled with ¹²⁵I (10⁶ cpm/ml).

Heat-inactivation of DEN-1 virus

DEN-1 virus from infected C6/36 mosquito cell culture supernates (approximately 4 × 10⁶ PFU/ml in Eagle's minimal essential medium with 2% bovine serum albumin) was heated at 56°C for 30 min. An aliquot of the heated virus was tested for residual viability by inoculating it onto C6/36 cell monolayers and plaquing on LLC cells. No plaques were observed. Antigenicity of the heated virus preparation was demonstrated by radio-immunoblot assay with DEN-1 virus hyperimmune mouse ascitic fluid. This heat-inactivated, whole virus preparation was used as a positive control immunogen in mice (see below).

Immunization of mice with recombinant antigen

To prepare recombinant antigen for mouse immunization, *S. frugiperda* cells infected with recombinant baculovirus were pelleted, lysed by Dounce homogenization, nuclei were removed by centrifugation at 1,000 × g, and the microsomal membrane fraction containing E antigen was pelleted at 100,000 × g for 1 hr, and resuspended in phosphate-buffered saline (PBS) (crude membrane preparation). Three-week-old BALB/c mice (Charles River, Wilmington, MA) were immunized by intraperitoneal (ip) injection with 0.1 ml of crude membrane preparation containing recombinant E antigen in PBS, pH 7.4, and intramuscular (im) injection with 0.1 ml of the same preparation in Freund's complete adjuvant (1:1 v/v) (first dose, day 1) and Freund's incomplete adjuvant (second dose, day 3, third dose, day 28, and fourth dose, day 30). Approximately 4 µg of E antigen was given in each dose. One day after the fourth dose, ascitic fluid formation was induced by ip injection of 5 × 10⁶ sarcoma 180 cells and fluids harvested from the peritoneal cavity about 12 days later with an 18 gauge needle.⁴⁰

Mouse protection assay

The BALB/c mice were immunized beginning at three weeks of age by ip and im injection with

0.1 ml of crude cell membrane preparation containing varying amounts of recombinant E antigen, an equivalent amount of membrane preparation without E antigen (negative control), or 0.1 ml of heat-inactivated DEN-1 virus (approximately 4×10^6 PFU/ml before inactivation; positive control). Injections were given ip without adjuvant and im in Freund's complete adjuvant (1:1 v/v) (first dose, day 1) and Freund's incomplete adjuvant (second dose, day 3, third dose, day 14). Mice were challenged on day 21 (at six weeks of age) with a lethal dose of Western Pacific strain (Nauru Island 1974) DEN-1 virus (50 μ l of a 1:100 dilution of virus-infected suckling mouse brain suspension, approximately 10^5 PFU, 100 LD₅₀) administered intracranially (ic). At each day post-challenge, the mice were observed for morbidity and mortality.

Radio-immunoblot assay

Culture supernates from DEN-1-infected C6/36 mosquito cells or uninfected (control) C6/36 cells were pelleted at 27,000 rpm in a Ti30 rotor for 3 hr at 4°C. The pelleted virus (antigen), concentrated about 30-fold, was resuspended in PBS and spotted onto nitrocellulose paper strips. Strips were blocked with 5% nonfat dry milk in PBS (blocking buffer) for 30 min at room temperature, washed three times with PBS, and incubated overnight at room temperature with ascitic fluids or sera (diluted 1:100 in blocking buffer) from immunized mice. Strips were then washed with PBS and incubated with goat anti-mouse immunoglobulin labeled with ¹²⁵I (10⁶ cpm/ml in blocking buffer) for 1 hr at room temperature. Strips were washed with PBS and counted in a gamma counter. Antisera giving cpm at least two times higher with infected cells compared with uninfected cells were considered positive (i.e., positive/negative, P/N ≥ 2).

Virus plaque-reduction neutralization (PRNT-50%) assay

Plaque reduction neutralization assays were done essentially as previously described,⁴¹ with ascites or sera from immunized mice and Western Pacific (Nauru Island 1974) DEN-1 virus. Monkey kidney (LLC-MK2) cell monolayers in 6-well plates (Costar, Cambridge, MA) were used. Plates were incubated in sealed plastic bags at 36°C in a CO₂ incubator. Plaques were visualized

by staining on day 7 with 0.05% neutral red in normal saline. Titers reported are the reciprocal dilutions of antisera that inhibited the number of virus plaques by $\geq 50\%$, compared with sera from mice immunized with PBS or with uninfected *S. frugiperda* cells. Titers ≥ 10 were considered positive.

Statistical analysis

Data were tested for significance using a Chi-square test, Fisher's exact test, or linear regression analysis, as appropriate.

RESULTS

Antigenicity and yield of recombinant protein from baculovirus-infected S. frugiperda cells

A DEN-1 E gene-specific cDNA fragment was cloned into baculovirus as shown in Figure 1. This fragment encodes 416 amino acids, or 83% of the E protein, and 36 amino acids of the M/prM protein, which precedes E in the genome and is required for membrane translocation of E.¹¹ The recombinant baculovirus was used to infect *S. frugiperda* cells. Lysates from cells infected with the recombinant baculovirus, wild-type baculovirus, or uninfected cells were electrophoresed on polyacrylamide-SDS gels.¹⁸ Proteins were stained with Coomassie blue (Figure 2, left panel) or transferred to nitrocellulose and reacted with DEN-1 virus-specific mouse hyperimmune ascitic fluid (MHAF) in a Western blot assay (Figure 2, right panel). An antigenic band of the size expected, around 48 kiloDaltons (kD), was observed only with recombinant-infected cells, demonstrating that this antigen was DEN virus specific (Figure 2, right panel, lane C). A corresponding Coomassie-stained band with the same molecular weight was not seen with recombinant-infected cells, indicating a lower than expected level of expression. The amount of recombinant antigen was estimated to be about 1 mg per 10⁹ cells by comparing, on a Western blot, the recombinant-infected cell lysate with highly purified recombinant E protein of known concentration (unpublished data).

In order to demonstrate specificity, the recombinant antigen was reacted with several monoclonal antibodies, including ones specific for the E protein of DEN-1. Positive reactions were seen with anti-E, but not anti-NS1 monoclonal anti-

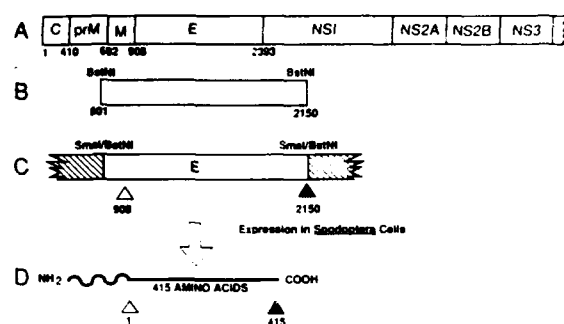


FIGURE 1. The E glycoprotein gene of DEN-1 virus expressed in recombinant baculovirus. A, DEN-1 virus cDNA, including the structural genes and the nonstructural genes up to NS3. B, *Bst*NI restriction fragment containing the recombinant E gene fragment. C, recombinant E gene fragment cloned at a *Sma*I site into the baculovirus polyhedrin gene (cross-hatched). D, recombinant protein expected after expression of the cloned gene in *S. frugiperda* cells. The recombinant construct contains 107 nucleotides derived from the 3' end of the prM/M protein gene encoding a hydrophobic transmembrane peptide (~), which is cleaved by host cell signal peptidase in dengue-infected cells, and extends through the first 1,245 nucleotides (415 amino acids) of the E gene. The first amino acid of the E protein and the putative signalase cleavage site is shown by the open triangle. The recombinant E protein was terminated 243 nucleotides (81 amino acids) before the 3' end by a UAA stop codon provided by the baculovirus vector (solid triangle). Nucleotide coordinates of the DEN-1 genome are from Mason and others.¹²

bodies (Table 1). These results confirmed that the recombinant antigen was a specific product of the cloned DEN-1 E gene.

The recombinant antigen was found to be intracellular and was not found secreted into the extracellular medium. The antigen was pelleted at $100,000 \times g$ for 1 hr. conditions which pellet intracellular membranes. This pelleted material, hereafter called the crude membrane preparation, was resuspended in PBS and used to immunize mice.

Immunogenicity of the baculovirus-recombinant antigen in mice

The recombinant E antigen was tested for its ability to stimulate antibodies against DEN-1 virus in mice. One group of five three-week-old BALB/c mice was immunized with a crude membrane preparation from recombinant baculovirus-infected *S. frugiperda* cells containing DEN-1 E antigen (four doses with 4 μ g of E antigen per dose). Another group of five mice was immunized with heat-inactivated DEN-1 virus

(4 doses with an estimated 1–10 ng of viral antigen per dose). A third group of five mice was injected with PBS without antigen. After the fourth immunization, mice were induced to make intraperitoneal ascites by injection of mouse sarcoma 180 cells.

Ascitic fluids obtained from each group of mice at 42 days post-immunization were pooled and tested for their ability to react with and neutralize the homologous strain of DEN-1 virus, Nauru Island 1974. The results are shown in Table 2. The DEN-1 virus-neutralizing antibody was detected by PRNT-50% assay in mice immunized with the recombinant antigen and in mice immunized with heat-inactivated DEN-1 virus. These mice also had antibody that reacted with DEN-1 virus antigen in a radio-immunoblot assay. Although the recombinant contained a small part of the M gene, antiserum from immunized mice did not react to M or prM proteins in a Western blot assay (unpublished data).

Protection of mice against lethal DEN-1 virus encephalitis by immunization with recombinant E antigen

The ability of recombinant E antigen to protect mice against mortality from DEN-1 virus encephalitis was then determined. One group of 10 three-week-old BALB/c mice was immunized with a crude membrane preparation from recombinant baculovirus-infected *S. frugiperda* cells containing DEN-1 E antigen (three doses with approximately 4 μ g of E antigen per dose). A second group of 10 mice was immunized with an equivalent amount of crude membrane protein from baculovirus-infected *S. frugiperda* cells that did not contain E antigen. A third group of 10 mice was immunized with heat-inactivated DEN-1 virus (four doses with an estimated 1–10 ng of viral antigen per dose). All mice were challenged at six weeks of age by ic injection of live DEN-1 virus. The mice were followed for 28 days, beyond which time no mortality due to virus infection was expected. Survival at each day post-challenge is shown in Figure 3. In this experiment, six of 10 recombinant-immunized mice ($P = 0.005$), and five of 10 mice immunized with heat-inactivated DEN-1 virus ($P = 0.02$) survived challenge. There were no survivors among mice that did not receive E antigen.

To determine if protection improved with increasing amounts of recombinant E antigen,

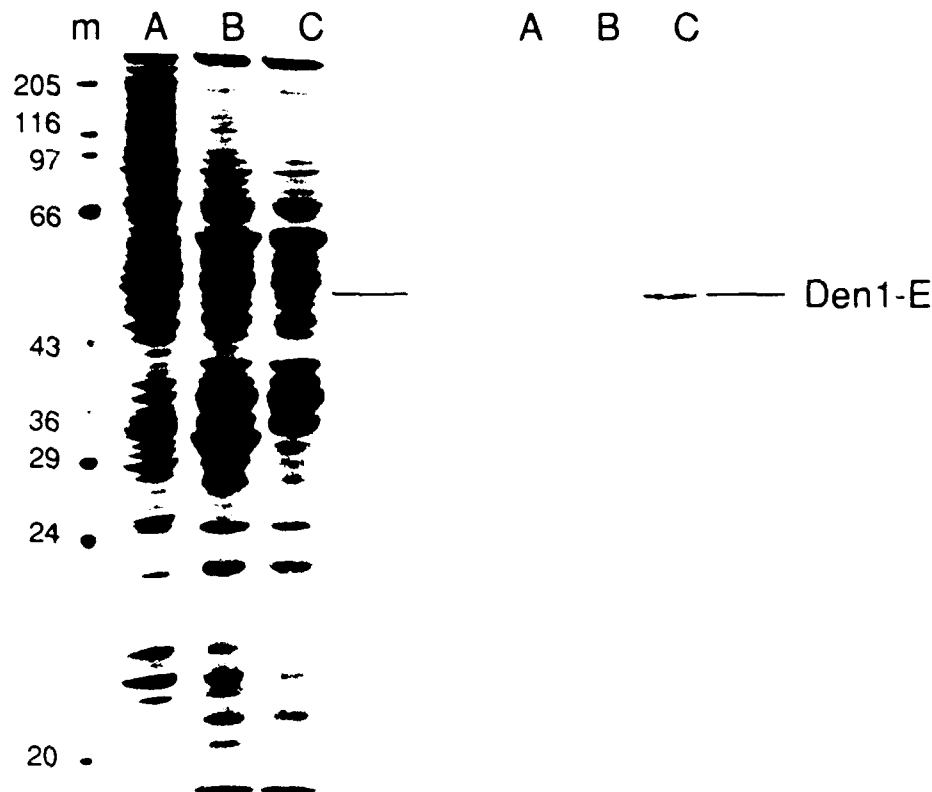


FIGURE 2. Antigenicity of the recombinant E protein. *S. frugiperda* cells were infected with recombinant baculovirus bearing the E gene of DEN-1 virus. Infected cells were lysed and electrophoresed on polyacrylamide-SDS gels. Separated protein bands were transferred to nitrocellulose and reacted with DEN-1 specific mouse hyperimmune ascitic fluid (MHAF) (right panel). Bands from a duplicate gel were stained with Coomassie blue (left panel). Lane A, uninfected cells; lane B, wild-type baculovirus-infected cells; lane C, recombinant-infected cells; lane m, molecular mass markers.

groups of 10 mice each were immunized with 0, 0.5, 3, 4, or 6.5 μ g per dose (three doses each). At six weeks of age, mice were challenged with live DEN-1 virus and observed for up to 28 days. Survival increased from two of 10 in the groups

that received 0.5 or 3 μ g per dose to eight of 10 in the group that received 6.5 μ g per dose (Figure 4). This represents a significant increase in survival from the lowest to the highest dose tested ($P = 0.01$). When percent survival was plotted

TABLE 1
Reaction of recombinant antigens with polyclonal and monoclonal antibodies^a

Antibodies	Recombinant antigens ^b	
	DEN 1 E	DEN 1 NS1
8C2	2.6	0.6
8B9	5.9	0.6
9D12	2.6	0.7
15F3	0.8	8.0
MHAF	3.3	4.6

^a Reactions of radio-immunoblot assay with results expressed as counts per min (cpm) with recombinant antigen divided by cpm with wild-type baculovirus control (Positive Negative, P.N.).

^b DEN 1 E antigen and NS1 antigen from recombinant baculovirus-infected *S. frugiperda* cell lysates diluted 1:100 in PBS.

Monoclonal antibodies 8C2, 8B9, 9D12 (E antigen-specific) and 15F3 (NS1 antigen-specific) diluted 1:250, and polyclonal mouse hyperimmune ascitic fluid (MHAF) diluted 1:500 in PBS.

TABLE 2
Antibody responses of mice multiply-immunized with baculovirus-recombinant and authentic DEN 1 antigens^a

Antibody to	PRNT-50 ^b	Immuno-blot (P.N.)
D1 E recombinant	20	11.3
Heated D1 virus	80	21.7
Live D1 virus (MHAF)	640	51.5
None	10	1.8

^a Serologic assays performed on pooled ascitic fluids from groups of 5 BALB c mice.

Plaque-reduction neutralization 50% endpoint titer (reciprocal antiserum dilution) against DEN 1 virus (Nauru Island 1974).

Radio-immuno-blot titer against DEN 1 virus antigen, P.N. = counts per min with infected cells/counts per min with uninfected cells; MHAF 1:500 dilution, other antibodies, 1:100.

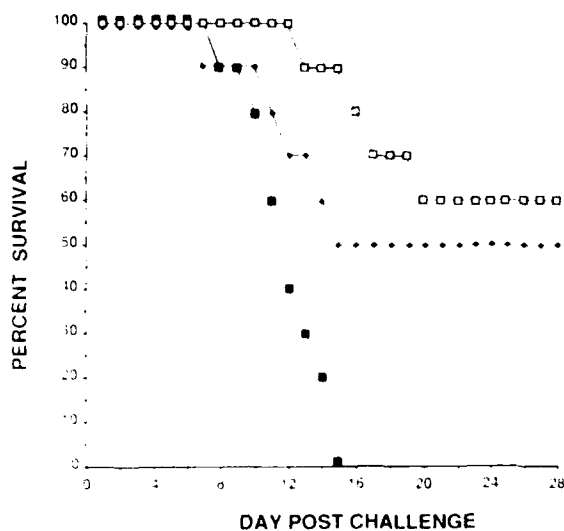


FIGURE 3. Survival of mice immunized with recombinant DEN-1 E antigen following challenge with DEN-1 virus. Groups of 10 mice each were immunized with baculovirus-recombinant DEN-1 E antigen ($4 \mu\text{g} \times 3$ doses) (□), an equivalent amount of protein from wild-type baculovirus-infected *S. frugiperda* cells that did not contain E antigen (■), or heat-inactivated DEN-1 virus ($1-10 \text{ ng of virus} \times 3$ doses) (●). Following immunization, mice were challenged by ic injection with approximately 100 LD_{50} of live DEN-1 virus. The percent of mice surviving at each day post-challenge is shown.

as a function of antigen dose, a linear regression coefficient (r^2) of 0.9 ($P = 0.02$) was obtained, indicating a positive correlation between antigen dose and survival.

DISCUSSION

In the present study, a 1,350 nucleotide DEN-1 cDNA restriction fragment that encodes the first 415 amino acids or 83% of the complete E protein was expressed in a recombinant baculovirus vector. Protein of the expected size, approximately 48 kD, was produced. The recombinant protein was antigenic with DEN-1-specific, polyclonal and monoclonal antibodies, and immunogenic in BALB/c mice, in which it stimulated the production of DEN-1 virus-neutralizing antibody. Immunized mice were significantly protected against lethal challenge with DEN-1 virus.

The DEN-1 virus used in the neutralization and challenge experiments was a Western Pacific isolate, Nauru Island 1974, the same as that used to make the cDNA expressed in baculovirus. Although protection against other strains of DEN-

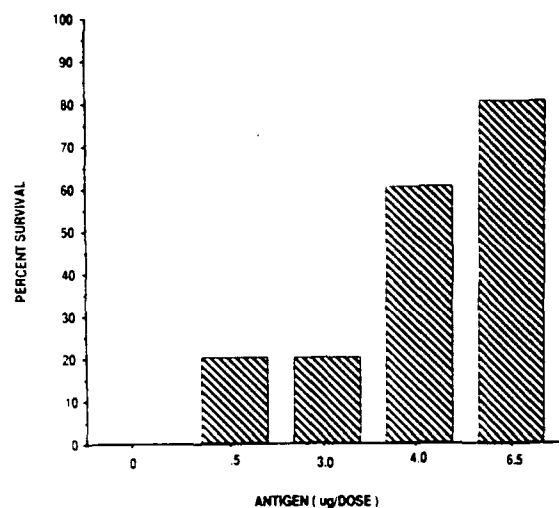


FIGURE 4. Effect of dose of recombinant E antigen on survival of mice challenged with DEN-1 virus. Groups of 10 mice each were immunized with different amounts of recombinant E antigen (0, 0.5, 3, 4, or $6.5 \mu\text{g per dose} \times 3$ doses), then challenged by ic injection with approximately 100 LD_{50} of DEN-1 virus and followed for up to 28 days. The percent of each group that survived virus challenge is shown.

1 was not tested, it is believed that this would have been achieved since all known DEN-1 virus strains appear to be cross-neutralizable.

The finding that recombinant DEN-1 E antigen reacted with E-specific monoclonal antibodies and elicited virus-neutralizing antibody is significant for two reasons. First, it demonstrates that recombinant and authentic E proteins share common neutralization epitopes. Second, virus neutralizing antibody has been found to correlate with protection against disease in a number of previous studies: mice immunized with a baculovirus recombinant JE E protein made neutralizing antibody and were protected against encephalitis.¹⁴ Similarly, mice given vaccinia expressing JE E antigen made neutralizing antibody and were strongly protected.⁴²

Passive administration of DEN-2 virus-neutralizing monoclonal antibodies was observed to protect mice against lethal virus challenge.²⁷ Conversely, a baculovirus recombinant DEN-1 E antigen that was ineffective at eliciting neutralizing antibody failed to protect mice (R. Putnak, unpublished data). Instead of containing 83% of the E protein, as in the present construct, this non-protective construct contained 100% of the E protein. Recently, Men and others⁴³ also reported that vaccinia-DEN 4 recombinants, truncated at the carboxyl-terminus to synthesize ap-

proximately 80% of the E protein, were more immunogenic in mice than recombinants that synthesized 100% of the E protein. These investigators speculate that truncated E protein is more immunogenic because it is more efficiently transported to the cell surface and secreted than full-length E protein. In the case of baculovirus-expressed DEN-1 E, both 83% and 100% proteins were prepared from lysed cells; this suggests that there may be other reasons for the superior immunogenicity of the 83% DEN-1 E protein.

The results also showed that increased protection of mice was achieved with higher doses of recombinant antigen. Because of the rather low level of expression, it was not practical to administer more than about 20 μ g per mouse. Although this dose conferred significant (80%) protection, it did not appear to be as effective on a weight basis as inactivated, whole virus, in which 3–30 ng protected 50% of mice. Thus, although baculovirus may allow for production of large quantities of recombinant protein, this advantage may be partially offset by lower immunogenicity of recombinant as compared with authentic protein.

Nevertheless, the results presented here appear to validate the use of baculovirus vectors for production of recombinant DEN-1 immunogens. We are currently attempting to extend these results to other DEN serotypes as well. We are also working to purify recombinant E proteins and improve expression level and immunogenicity with the goal of developing candidate DEN subunit vaccines.

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Authors' addresses: Robert Putnak, Robert Feighny, Jeanne Burrous, and Charles Hoke, Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, DC 20307-5100. Craig Hackett and Gale Smith, MicroGeneSys Inc., 400 Frontage Road, West

Haven, CT 06516. Mark Cochran, Molecular Therapeutics, 400 Morgan Lane, West Haven, CT 06516.

Reprint requests: Robert Putnak, Department of Virus Diseases, Building 40, Room 2036, Walter Reed Army Institute of Research, Washington, DC 20307-5100.

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